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METHODS TO IDENTIFY WATERBORNE PATHOGENS AND INDICATOR ORGANISMS

by

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16. ABSTRACT

The number of methods described were selected because they have been successfully used to monitor water quality or to detect pathogens associated with water contact diseases. Some are included because they have characteristics that fulfill a unique purpose, i.e., high volume enumeration techniques for measuring bacteria that normally occur in water at very low densities. The bacterial indicator methods, if used properly, are an effective means of monitoring water quality and thereby protecting groups at risk against pathogens associated with fecal material from humans and other warm-blooded animals. The methods that isolate frank and opportunistic pathogens from water samples are most useful for identifying the source of these organisms during disease outbreaks associated with water contact. This report contains a 127 reference bibliography.

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I. INTRODUCTION

The microbiological methods used to enumerate and isolate bacteria from water samples obtained from aquatic environments and potable water fall into two categories. One group of methods measures indicator bacteria to signal the potential presence of pathogens or undesirable microorganisms in water. The classic bacterial indicators are those that indicate the presence of fecal contamination. Early microbiologists were aware of the difficulties associated with measuring or detecting enteric pathogens in water. They reasoned that by isolating an innocuous bacterium, consistently found only in feces, from water, they could assume the presence of fecal material and hence, the possible presence of enteric pathogens. Indicator bacteria are usually harmless, occur in high densities in their natural environments and are easily cultured on relatively simple bacteriological media. Most of the enteric pathogens that were impossible or difficult to isolate by the early microbiologists can now be cultured, but the philosophy of using indicators has not changed. The reason for this is that pathogens usually occur sporadically in the water environment and the isolation of one pathogen will not necessarily predict the presence of another pathogen. Furthermore, many enteric viral pathogens, such as rotavirus and Norwalk Agent, are either difficult to culture or unculturable. Thus, the concept of using indicator bacteria is as useful today as it was near the turn of the century when Escherich first proposed their use.[1]

The second category involves methods which isolate pathogens or opportunistic pathogens from aquatic environments and potable water systems. The methods in this category usually are modifications of methods developed in clinical laboratories for isolating pathogens from patient specimens. The isolation of pathogens from water is frequently difficult and cumbersome

because of interferences by natural competing flora and the fastidious nutrient requirements of the pathogens. Enrichment procedures must be used in some cases before the organisms are transferred to harsh selective media. The need to enrich obviously limits the ability of some methods to quantify the pathogens in the sample.

Methodology for viral isolation will not be addressed in this chapter since many of the important viral pathogens are difficult or impossible to culture from water samples. Furthermore, the sporadic nature of the occurrence of viral pathogens and the logistical complexity of routine monitoring virtually precludes measuring the culturable viruses on a systematic basis.

II. ISOLATION AND ENUMERATION METHODS FOR INDICATOR BACTERIA

A. Total Coliforms

The coliforms[2] are a group of bacteria defined as gram-negative, aerobic or facultatively anaerobic, nonspore-forming rods that ferment lactose in 48 hours at 35°C with gas production by the most probable number (MPN) methodology. Coliforms were originally believed to indicate the presence of fecal pollution, and they are still used for that purpose. Coliform testing also is prescribed for determining the sanitary integrity of potable water in distribution systems, even though some coliforms have been found to be widely distributed in nature and are not associated with the intestinal tract of warm-blooded animals.[3] This finding has limited the use of coliforms as indicators of fecal pollution, in surface waters, however, they are still widely used as indicators of potable water quality in the United States.

The methods[2] approved by the U. S. Environmental Protection Agency for regulatory purposes and most commonly used to enumerate

coliforms are the lactose broth or Lauryl Sulfate Tryptose (LTB) broth MPN method and the membrane filter technique employing m-Endo broth or agar.

Another membrane filter method, the mC method,[4] has been reported for enumerating coliforms and their component genera from seawater. Confirmation of typical coliform colonies on this media from marine water samples was 95 percent. A comparison of marine water samples analyzed by this method and the coliform MPN and m-Endo methods showed that in 14 of 20 samples recovery of coliforms by the mC method was greater than with the m-Endo method. The mC and MPN method were comparable in their recovery of coliforms. A new method, using m-T7 medium,[5] has been reported to recover higher numbers of injured coliforms than currently recommended methods. The new method recovered an average of 43 percent more verified coliforms from drinking water samples than the m-Endo technique.

LeChevallier et al.[6] have proposed new verification tests for coliforms from membrane filter methods. These tests would substitute for the subculturing of colonies to Lactose broth and subsequent transfer of gas positive culture tubes to brilliant green bile broth. These investigators indicated that the new verification procedure increased the confirmation rate by 87 percent over the currently accepted methods for membrane filter coliform colonies obtained from drinking water.

Total coliforms are used to measure the quality of potable water, shellfish harvesting waters and, in at least 23 percent of the states, to determine the quality of recreational waters. The finding of coliforms in these different types of waters is interpreted as a degradation of the quality of the water, and therefore, a

potential health hazard to users or to consumers of shellfish harvested from the water. This interpretation has severe limitations, however, because one member of the coliform group, Klebsiella, has been shown to grow in industrial and agricultural wastes which are unrelated to fecal contamination.

B. Fecal Coliforms

Fecal coliforms[2] are a subgroup of the total coliform group which can be differentiated by their ability to grow at an elevated temperature (44.5°C ± 0.2°C). This group of organisms is measured with the EC broth MPN procedure or the mFC membrane filter method. The fecal coliforms can comprise four genera of bacteria, Escherichia, Klebsiella, Citrobacter and Enterobacter, some of which are capable of thermotolerant (44.5°C) growth. The thermotolerant characteristic separates the fecal coliforms, which may be found in the gastrointestinal tract of warm-blooded animals, from the other coliforms.

Rose et al.[7] proposed a two-layer method for enumerating fecal coliforms which yielded higher recovery than existing fecal coliform membrane filter procedures. This procedure was evaluated using raw wastewater, chlorinated wastewater, fresh surface water and marine waters. Fecal coliform colony verification was 92 percent and comparison of the two-layer method with existing methods showed that the new method was more efficient for recovery of fecal coliforms in these waters. Presswood and Strong[8] reported a possible toxic effect of rosolic acid used in mFC medium. They observed that the use of mFC medium without rosolic acid resulted in higher counts 71 percent of the time compared to mFC containing rosolic acid, in

water samples from unchlorinated and chlorinated sewage, and fresh surface waters. Verification of typical blue fecal coliform colonies from the modified medium had a fecal coliform verification rate of about 94 percent.

Lin[9] has described a two-step membrane filter method utilizing a phenol red lactose broth pre-enrichment technique followed by transfer of the filter to mFC agar incubated at 44.5°C that was very effective for detecting fecal coliforms in chlorinated sewage effluents. He showed that this method was comparable to the EC MPN method when both were used to assay 126 chlorinated water samples. Green et al.[10] reported that recovery of fecal coliforms from chlorinated sewage effluents by the mFC technique was improved by first incubating the medium at 35°C for 5 hours followed by incubation for 18 hours at 44.5°C.

Stuart et al.[11] compared a two-layer membrane filter medium (IM-MF) for injured fecal coliforms with the EC MPN procedure and the standard mFC method using various types of water samples. The IM-MF method recovery of fecal coliforms was equal to or greater than the MPN technique recoveries in 50 percent of the samples tested and always greater than the mFC method. The IM-MF method exceeded the standard method in 30 of 33 trials using stream water samples.

LeChevallier et al.[12] evaluated m-T7 agar as a fecal coliform medium using sewage effluent samples. The m-T7 medium was compared to the standard mFC method and the two-layer enrichment procedure of Rose et al.[7] Higher fecal coliform counts were obtained with the m-T7 medium than with the other methods tested. Verification of

isolated fecal coliforms averaged 89 percent for M-T7 and 83 percent for mFC. The highest fecal coliform isolation rates were observed with sewage effluents when m-T7 was preincubated at 37°C for 8 hours followed by transfer to 44.5°C for 12 hours.

The presence of fecal coliforms in water is indicative of fecal contamination and the possible presence of enteric pathogens. Thus, finding fecal coliforms in samples of recreational water or shellfish harvesting water indicates that water or shellfish are contaminated by fecal pollution or that swimming in the water or consuming shellfish harvested from the water may result in acute illness.

C. Escherichia coli

E. coli is an unquestionable inhabitant of the gastrointestinal tract of man and other warm-blooded animals. It is unable to show sustained growth in water or other extra-intestinal environments. This organism, which is a member of the well-known fecal coliform group, is easily distinguished from other genera of the fecal coliform group, by the urease test. E. coli does not possess this enzyme, whereas it is possessed by other coliforms, such as Klebsiella, Enterobacter and Citrobacter. This characteristic was used by Dufour et al.[13] to develop a membrane filter method that was 90 percent specific for E. coli from marine, estuarine and fresh water samples. The method (mTEC) used a lactose base medium which is incubated at 44.5°C to select for thermotolerant coliforms and those coliforms able to grow at this temperature were differentiated using an in situ urease test.

The finding of E. coli in water samples assayed with the mTEC procedure is clear evidence of fecal contamination of the water from

which the sample was taken. Furthermore, at freshwater beaches, the density of E. coli in the water has been shown to be directly related to the incidence of swimming-associated illness.[14] This relationship is limited to freshwater, since Cabelli[15] has reported that this indicator organism does not relate very well to swimming-associated illness in marine waters.

D. Fecal Streptococci

The fecal streptococci[2] are comprised of Streptococcus faecalis, S. faecalis subsp. liquefaciens, S. faecalis subsp. zymogenes, S. faecium, S. bovis, S. equinus and S. avium. All of these streptococci are members of Lancefield's Group D Streptococci. The fecal streptocci have long been proposed as indicators of fecal contamination since most of them are found in the gastrointestinal tracts of man, domestic and feral animals, and birds. However, there are reports that some of the fecal streptococci may be found on vegetation and insects.[2]

The fecal streptococci do not reproduce in the environment, but may survive for prolonged periods. They are less dominant in the feces of many animals, birds, etc. than other gastrointestinal bacterial flora such as members of the coliform group.

Three methods used to enumerate the fecal streptococci group are the azide dextrose (AD) MPN multitube method, the KF membrane filter technique and the Pfizer Selective Enterococcus agar (PSE) pour plate procedure.

The AD multitube MPN method involves incubation of azide dextrose broth, to which measured quantities of the water sample have been added, at 35°C for 24 to 48 hours. Inoculum from tubes showing

turbidity after 24 and/or 48 hours, is streaked onto PSE agar plates. The plates exhibiting typical colonies with esculin hydrolysis are positive for fecal streptococci. MPN tables are used to estimate the number of streptococci in the water sample. The direct plate count methods may be used for samples with high turbidity. KF agar, PSE agar or esculin-azide agar can be used as pour plate media for turbid water samples. The membrane filter method involves filtration of the water sample followed by incubation on KF streptococcus agar medium at 35°C for 48 hours.

Typical streptococci isolated with these methods may be confirmed by the following tests: presence of catalase; growth in BHI broth incubated at 45°C in 48 hours; growth in 40 percent bile broth medium incubated at 35°C for 72 hours. The fecal streptococci may be diffferentiated further by the methods described by Facklam,[16] Gross et al.[17] or Watkins et al.[18]

Pavlova et al.[19] compared five media, M-enterococcus agar, KF agar, azide-sorbitol agar, thallous-acetate agar and PSE medium for the isolation and enumeration of fecal streptococci from animal and bird feces, raw sewage and foods. These investigators determined that KF and PSE agar were essentially equal in their ability to enumerate fecal streptococci, but PSE required only 24 hours of incubation. Brodsky and Schiemann[20] compared PSE and KF media membrane filter procedures for the recovery of fecal streptococci from sewage effluent. They reported that PSE medium was highly specific for enterococci (86 percent) and fecal streptococci (90 percent) and that KF medium was less selective for fecal streptococci (83 percent). KF broth, PSE agar, mSD agar, Slanetz-Bartley broth (35°C)

and Slanetz-Bartley broth (4 hours at 30°C and then 44 hours at 44°C) were compared for the enumeration of fecal streptococci from sewage by Stanfield et al.[21] They reported that KF was the least selective method tested. It is suggested that PSE or Slanetz-Bartley medium at 35°C is best for the enumeration of Group D streptococci from sewage.

Dutka and Kwan[22] compared eight media for the recovery of fecal streptococci from unchlorinated sewage effluent, sewage lagoon effluent and fresh surface waters. The media compared were m- Enterococcus agar, KF agar, PSE agar, mE plus EIA agar and AD-EVA broth. Several of these methods involved using different incubation temperatures. These investigators reported that an incubation temperature of 44.5°C did not enhance the selectivity of any of the media. The most efficient membrane filter methods tested were PSE agar, KF agar and mE agar (41°C, 48 hours). The AD-EVA MPN procedure at 35°C was as efficient as the most efficient membrane filter method. Pagel and Hardy[23] compared PSE agar, KF agar, mEnt agar, mE-EIA and Slanetz-Bartley (SB) media, for the recovery of fecal streptococci from natural sources. They concluded that PSE agar was the least inhibitory, KF agar had the lowest specificity and the best overall medium was mEnt agar.

The MPN and pour plate methods are limited to the use of relatively small volumes of water samples. The membrane filter methods on the other hand allow large volumes of water samples to be tested, and they are more precise. The literature indicates that without confirmation, all of the media are limited in their ability to accurately enumerate fecal streptococci.

The presence of fecal streptococci in water is an indication of fecal pollution and the potential presence of gastrointestinal pathogens, but this interpretation is limited by similar considerations as finding coliforms.

E. Enterococci

The enterococci are a subgroup of the Group D fecal streptococci and include the genera, Streptococcus faecalis, Streptococcus faecium and Streptococcus avium.[24,25] The members of this group commonly inhabit the intestinal tract of humans and warm-blooded animals, and therefore, they have long been used to detect fecal contamination of water. Several media have been developed to isolate and enumerate enterococci using membrane filter methods. Slanetz and Bartley[26] developed mEnterococcus agar for detecting enterococci in water, sewage and feces. Doust and Litsky[27] modified the Pfizer Selective Enterococcus (PSE) agar developed by Isenberg et al.[28] for use as a membrane filter method and Levin et al.[29] developed mE agar for isolating enterococci from marine waters. Stanfield et al.[21] compared the growth of various species of streptococci on these three media and found none of them to be specific for enterococci. The mEnterococcus agar supported the growth of Streptococcus bovis, Streptococcus equinus, Streptococcus mitis and Streptoccus salivarius, as well as the enterococci strains. The S. bovis and S. equinus also were able to grow on PSE agar in addition to the enterococci. The only non-enterococcus species able to grow one mE medium was S. equinus. Although none of the media are specific for enterococci, in practice very few fecal streptococci that are not enterococci are enumerated.

Pagel and Hardy[23] compared the specificity of these three media using raw sewage, primary sewage effluents and storm sewer effluents. S. bovis was identified twice on PSE agar and once on mEnterococcus agar and not at all on ME agar. S. salivarius was identified once on mEnterococcus agar. Stanfield et al.[21] found the mE method to be the most specific of the three methods followed by mEnterococcus agar and then PSE agar, whereas Pagel and Hardy[23] found the PSE method to be most specific followed by the mE and mEnterococcus methods. Dutka and Kwan[22] examined various types of water samples using many methods including the three methods described above. They ranked the methods according to their ability to isolate enterococci and found that it followed the order mE agar, PSE agar and mEnterococcus agar. The results of these three studies[21,22,23] indicate that, overall, mE medium probably has the highest specificity for enterococci. The ability of this medium to selectively isolate enterococci from water samples and wastewater effluents was a major reason for its being used during the health effects recreational water quality studies conducted by Cabelli et al.[30,31] The organisms enumerated by this method showed the best relationship to swimming-associated gastroenteritis in marine and fresh water swimmers. The finding of these enterococci in fresh or marine surface waters, as measured by the mE method, can be directly translated to a rate of swimming-associated gastroenteritis using the relationship reported by Cabelli[15] and Dufour.[14]

III. ISOLATION AND ENUMERATION METHODS FOR PATHOGENS AND OPPORTUNISTIC PATHOGENS

A. Salmonella

A great deal of emphasis has been placed on the presence of Salmonella species in water environments, because of their historic relationship to waterborne and water contact disease. Although the incidence of illness caused by Salmonella has dramatically decreased in the United States since the mid-twentieth century because of good hygiene and sewage treatment practices, as well as, the availability of antibiotics to treat patients with the disease, its presence in water must still be considered a threat to public health. Methods for the detection and isolation of this genus are important for identifying possible sources of the organism in disease outbreaks associated with water environments.

Most methods for isolation of this organism from water samples employ concentration techniques because of the low densities at which Salmonella occur in water. Filtration through membranes or diatomaceous earth, followed by incubation of the filter material or membranes in an enrichment medium, i.e., Dulcitol Selenite or Tetrathionate broth, and the subsequent streaking of inocula from the enrichment broth to several selective plating media, such as Brilliant Green Agar, Bismuth Sulfite Agar, Xylose Lysine Desoxycholate agar is the most commonly used method for isolation of Salmonella. Isolated typical Salmonella colonies are then identified by biochemical and serological techniques.

The methods described by Grunnet[32], Cheng et al.[33] and Levin et al.[34] have been developed to concentrate and enumerate Salmonella in water samples. Methods which use adsorbent pads suspended in water for long periods of time have also been used to concentrate Salmonella from water.[35]

The procedure described by Cheng et al.[33] is stated to give results in 48 hours and is based on the use of specific antisera in a mannitol medium for identification of Salmonella. However, problems associated with serological flocculation have been noted with this technique.

The method described by Levin et al[34] requires the use of fiberglass epoxy depth filters, followed by enrichment and selective plating. The use of these filters and their accompanying apparatus may be cumbersome and expensive, however, this method has been successful for enumerating low levels of Salmonella in marine waters.

A facile, definitive method for the isolation and enumeration of Salmonella in water samples is not available, but the methods described above have been quite adequate, especially for detecting Salmonella associated with waterborne outbreak episodes.

B. Shigella

The Shigella genus consists of four species,[36] S. dysenteriae, S. flexneri, S. boydii and S. sonnei. This pathogen can be present in the intestinal tract of infected humans, and therefore, the transmission of shigellosis usually is fecal-oral in nature. This organism is a of the leading causes of diarrhea in humans.[36] The presence of Shigella in any type of water represents an immediate human health risk to individuals using the water. This pathogen has been implicated in waterborne outbreaks[36] and in an outbreak of shigellosis among bathers swimming in contaminated river water.[37]

The methodology for the isolation of Shigella from water has been adopted from techniques primarily used for clinical samples and the success of using these techniques in isolating this organism from

natural waters has been varied. The organisms can be concentrated from water by a variety of techniques.[2] After concentration of the water sample, enrichment techniques are employed, followed by plating on selective media and biochemical or serological identification.

Several reports[38,39,40] have indicated that GN (Gram Negative) broth is the enrichment medium of choice for the isolation of Shigella from feces. These same investigators, including Morris et al.,[41] report that the best selective plating medium for isolating Shigella from enrichment broths is XLD agar.

Park and his colleagues[42,43] introduced a novel technique for selecting Shigella from water samples. They incorporated 4-chloro-2-cyclopentylphenyl-B-D-galactopyranoside, an analogue of lactose, into lactose broth. Bacteria that utilize lactose, such as coliforms, hydrolyze the compound to galactose and a toxic moiety which subsequently kills them. Shigella, which do not uitlize lactose, are not affected. These investigator's reported that 42 of 48 Shigella strains were selectively enriched by this procedure. This selective enrichment procedure, followed by plating on a selective medium, could be employed as a modified MPN method for the isolation and enumeration of Shigella from water. This technique has not gained wide acceptance, probably because of the high cost of the lactose analogue.

Because of the sporadic nature of disease caused by Shigella species, it is not reasonable to monitor waters for this pathogen on a routine basis. However, in situations where an outbreak of shigellosis has occurred, it is mandatory that the suspected waters be examined to determine the source of the pathogen if at all pos-

sible. The detection of Shigella species in surface or potable waters, to which ill individuals have been exposed, should be interpreted as prima facie evidence of the source of the etiologic agent.

C. Yersinia enterocolitica

Yersinia enterocolitica has been implicated in many waterborne disease outbreaks.[44] Yersinia associated with disease and the environment recently have been divided into four species, Y. enterocolitica, Y. kristensenii, Y. frederiksenii and Y. intermedia, based on DNA-relatedness and biochemical reactions.[44]

Methodology[46] for the isolation of Y. enterocolitica from water has utilized media developed for other purposes. Traditionally, isolation of Yersinia has been accomplished by incubation of the water sample itself or the membrane filter through which a water sample has been passed in mild buffer or Cooked Meat Media (CMM) at low temperature (4°C) for up to 21 days. The water sample or medium containing the membrane filter are sampled at 7, 14 and 21 days by plating portions onto an agar medium.

Several methods recently have been developed which are more rapid and facile. The membrane filter method of Bartly et al.[47] is based on the ability of Y. enterocolitica to ferment sorbitol, the absence of the enzymes, lysine decarboxylase and arginine decarboxylase-dihydrolase and the presence of urease. Presumptive identification of Y. enterocolitica is made in approximately 48 hours. Confirmation[48] of the isolates are accomplished by biotyping and serotyping.

An enrichment broth for the isolation of Y. enterocolitica has been reported by Weagant and Kaysner.[49] This medium uses sorbitol as

the primary differential substrate. This broth medium may be inoculated directly or water samples may be filtered and the filters placed into the medium. The inoculated medium is incubated at 22°C for 48 hours. Portions from tubes of enrichment broth showing growth are inoculated onto an agar medium to obtain colony isolates. Isolates are identified further by biochemical and serological test procedures.

A selective differential plating media (CIN) based on mannitol fermentation and the use of antibiotics and bile salts for the suppression of competing bacterial flora has been described by Schiemann.[50] Although developed for the isolation of Y. enterocolitica from feces this medium may be useful for isolating this organism from water samples.

Head et al.[51] compared six Y. enterocolitica isolation media using 35 laboratory bacterial strains seeded into fecal specimens. They determined that CIN medium was the most efficent for inhibiting competing flora and supporting the growth of the target organism after 40 hours of incubation. The membrane filter method of Bartley et al.[47] isolated 33 strains of Y. enterocolitica from 15 of 27 river water samples. The frequency of isolation by the enrichment technique of Weagant and Kaysner[49] using natural samples of fresh and marine water recovered Yersinia in 22 of 24 (92%) and 95 of 127 (75%) samples, respectively. These investigators also reported the isolation of indigenous Y. enterocolitica from public drinking water systems by using epoxy-glass fiber filters followed by placing the inoculated filter into Peptone-Sorbitol-Bile Salts (PSB) enrichment media.

A very thorough review of the literature on Y. enterocolitica has been conducted by Swaminathan et al..[52] Water has been implicated as the common source in several outbreaks[44] involving Y. enterocolitica. Yersinia has been isolated from numerous waters, but the strains found in water usually differ from those found in human disease. Therefore, the significance of finding Y. enterocolitica and Y. enterocolitica-like organisms in water is not known at this time. Unless organisms are isolated during a waterborne outbreak or the isolates are members of those serotypes known to cause disease in humans, it is unlikely that the isolation of organisms from aquatic environments pose a significant health risk for humans.

D. Campylobacter

Campylobacter jejuni recently has become recognized as one of the most common causes of bacterial gastroenteritis.[53] This organism is commonly found in the intestinal tracts of domestic and feral animals and in birds. These animals apparently act as the reservoir of this organism which is transmitted to humans through direct contact or indirectly through contaminated water, milk or food.[54]

C. jejuni produces gastroenteritis in healthy and impaired persons at relatively low levels[55] and has been implicated as the etiologic agent of gastroenteritis outbreaks in Sweden and the United States associated with municipal water supplies. It is generally believed that this organism normally is found in waters which contain fecal coliforms, which clearly suggests that feces contaminated water is a risk factor for Campylobacter gastroenteritis.

The methods currently used for the isolation of this pathogen were initially developed for isolating Campylobacter from clinical fecal specimens. Using these methods, Campylobacter has been isolated from marine and fresh waters.[56,57,58] The methods involve filtration of the water sample, followed by enrichment in broth medium or placing the filter on a selective media, with subsequent incubation under microaerophilic conditions at 42°C. The organisms usually require further purification for identification purposes. One of the basic limitations of these methods is their inability to prevent the spreading and coalescing of colonies.

A selective medium for the isolation of Campylobacter was reported by Bolton and Robertson called Preston medium.[59] Included in the report was an enrichment medium containing the same ingredients as Preston agar but without the agar. Preston medium was compared with Skirrow's medium for the isolation of Campylobacter from the feces of humans, cattle, pigs, sheep, chickens and seagulls. The Preston medium isolated more Campylobacter than the Skirrow medium and if the Preston enrichment medium was used considerably more Campylobacter isolates were found. These investigators also reported that the enrichment broth and Preston agar medium have been used successfully for two-and-one-half years for the isolation of Campylobacter from coastal seawater, drain swab effluents and abattoir environmental specimens.

Ribeiro et al.[60] have reported the successful use of the Preston medium enrichment method for the isolation of Campylobacter from human feces and domestic pets. They also reported that membrane filtration of water sample followed by placing the mem-

brane in Preston enrichment medium and subsequent subculture to a selective medium resulted in 12 positive out of 64 water samples examined. All of these water samples were negative using direct plating techniques.

Bolton et al.[61] have reported the use of Preston enrichment broth followed by subculture to Preston agar medium and reported that this method was capable of estimating as few as 10 Campylobacter per 100 ml of water. Using their method, 11 of 49 marine water samples were Campylobacter positive and counts ranged from 10 to 230 per 100 ml. Positive river water samples contained 10 to 36 Campylobacter colonies each in 7 of 44 samples. They also reported that by using a larger volume of water sample and membrane filters that an additional 24 of the "negative" samples were found to contain Campylobacter.

The Preston enrichment technique was tested further by Ribeiro and Price[62] and they reported that of 128 water samples tested, 34 were positive using this technique. Bolton et al.[63] compared Skirrow's, Butyle's, Blaser's Campy-BAP and Preston media for Campylobacter isolation from human, animal and environmental specimens. Preston enrichment broth followed by subculture to Preston agar gave the highest isolation rates.

Campylobacter isolates obtained by the Preston enrichment technique can be further identified by the procedures of Skirrow and Benjamin[64] and Kaplan.[65]

Mathewson et al.[66] evaluated three microporous filters and three pore sizes of a depth filter. They determined that a positive charged depth filter gave the highest percent recovery using seeded

sterile tap and surface waters. This type of methodology could possibly be used to enhance Campylobacter recoveries when using the filter enrichment MPN technique.

Another enrichment technique has been reported by Oosterom et al.[67] for detecting Campylobacter in sewage and river water samples. However, very little supporting information concerning this methodology is available.

The presence of Campylobacter jejuni in water indicates recent fecal contamination and the risk of infection for individuals using the water.

E. Pseudomonas aeruginosa

Pseudomonas aeruginosa has frequently been implicated as the causative agent for infections and illnesses associated with water contact. Two methods have been proposed for isolating this organism from water environments. Drake[68] proposed using a liquid medium in conjunction with the most probable number (MPN) technique. The medium has been used to quantify P. aeruginosa in feces[69] and in river and sewage samples.[70] A modification of Drake's method also has been recommended in the 15th edition of Standard Methods for the Examination of Waters and Wastewaters.[2] This method, however, has all of the shortcomings commonly identified with MPN procedures. Confirmation of questionable positive tubes is usually required, other microorganisms may interfere with the growth of P. aeruginosa, the volume of sample that can be assayed is limited and the technique itself is very imprecise. Many of these shortcomings were eliminated by the membrane filter method developed by Levin and Cabelli[71] for quantifying P. aeruginosa. The medium used for this method is highly

selective, and it incorporates a differential dye system that is specific for P. aeruginosa.

The significance of finding P. aeruginosa in surface waters is not clear. Foster et al.[72] attempted to relate the density of this organism to the incidence of ear infections among bathers. They were unable to demonstrate a relationship between P. aeruginosa densities and the rate of ear infections. Calderon and Mood[73] observed similar results in swimming pools. They found that the water quality of swimming pools as measured by the presence of P. aeruginosa was not associated with cases of otitis externa. Seyfried and Frazer,[74] on the other hand, did find a relationship between the presence of P. aeruginosa in swimming water and colonization of the outer ear and subsequent development of ear infections in swimmers.

It is unlikely, however, that Pseudomonas can be used to predict the incidence of otitis externa until a direct relationship between these two variables has been shown. Pseudomonas aeruginosa is the causative agent in many opportunistic infections linked to water contact, and it is in those cases that the methods described above will be useful for identifying the source of the pathogen.

F. Aeromonas

Aeromonas hydrophila shares many characteristics with the genera that comprise the coliform group. The fact that many members of this genus ferment lactose with gas production has drawn much attention to this ubiquitous organism because it may inflate coliform densities and give a false indication of poor quality water. It can easily be distinguished from coliforms because it possesses a cyto-

chrome oxidase that can be detected with a simple test.

Shubert[75] developed a medium for A. hydrophila (DSF medium) in 1967. DSF medium, which contains sodium sulfite and dextrin, was used in conjunction with a membrane filter procedure. In 1973, Shotts and Rimler[76] developed a selective-differential medium (RS) to isolate A. hydrophila. Sodium desoxycholate and novobiocin are the selective agents and aeromonads are differentiated from other organisms that grow on this medium with an indicator system based on H_2S production, maltose fermentation and ornithine decarboxylation. Fliermans,[77] in 1977, used the RS medium with membrane filters and found this technique to be effective. In 1979, Rippey and Cabelli[78] described a membrane filter enumeration method for A. hydrophila (mA). The medium employs trehalose as a fermentable carbohydrate, and ampicillin and ethanol as selective inhibitors. After 20 hours of incubation at 37°C, an in situ mannitol fermentation test followed by an in situ oxidase test is used to differentiate A. hydrophila from other competing organisms. This method was very successful in recovering A. hydrophila from fresh surface waters.

A. hydrophila is ubiquitous in freshwater environments, and therefore, detecting this organism in surface or potable water samples does not carry any special significance. The methods can be useful, however, for determining the source of the etiological agent of illness or infection.

G. Mycobacterium

The mycobacteria usually associated with water environments are Mycobacterium kansasii, Mycobacterium xenopi, Mycobacterium intracellulare and Mycobacterium marinum. Isolating mycobacteria from

water requires three steps. The first step involves concentration of the sample, either by centrifugation or filtration. This step is usually followed by a decontamination process whereby sodium hydroxide,[79,80,81] or a mixture of sodium hydroxide and sodium hypochlorite[82] or cetylpyridinium chloride[83] is added to the concentrate or directly to the water sample to eliminate unwanted competing bacteria. Bailey et al.[80] eliminated the decontamination step by applying their membrane filters directly onto plates containing selective medium.

The third step involves placing the concentrated sample or the membrane on a selective medium. Lowenstein-Jensen medium and Middlebrook's 7H10 medium have both been used to isolate mycobacteria from water samples. These media have been modified by various investigators who added antibiotics to the base formulations. Petron and Vera[84] added cyclohexamide, lincomycin and nalidixic acid to Lowenstein-Jensen or Middlebrook 7H11 media, and this modification was found to inhibit gram-negative bacilli while not affecting the recovery of mycobacteria. Lorian and Maddock[85] incorporated colimycin, spiramycin and amphotercin B into Middlebrook's 7H10 agar and found this to be an effective combination of inhibitors for decreasing contamination of their plates. Mitchison et al.[86] developed a selective 7H11 agar which contained polymyxin B, carbenicillin, amphotercin B and trimethoprim. McClatchy et al.[87] recommended that the concentration of carbenicillin in the selective 7H11 medium be reduced from 100 µg/ml to 50 µg/ml for better growth of M. intracellulare, M. kansasii and M. scrofulaceum.

The finding of mycobacteria in surface or potable waters is

probably not cause for taking steps to eliminate these organisms in the absence of overt disease in the exposed population. In those situations where outbreaks or infections are observed, isolation of mycobacteria from the water may be indicative that water is the vehicle of transmission and that appropriate water treatment procedures should be considered.

H. Vibrio

Vibrio parahemolyticus, Vibrio alginolyticus, Vibrio anguillaruim and Vibrio cholera are found in marine waters where they frequently cause disease in individuals who come in contact with the water. Thiosulfate-Citrate-Bile-Salt-Sucrose (TCBS) agar[88] is the most common medium used to isolate Vibrio species. High pH (8.6), bile salts and citrate inhibit some of the background flora found in marine waters, but the medium is frequently overwhelmed by growth of organisms such as Aeromonas species. Thus, follow-up tests are required to confirm Vibrio species. Akiyama et al.[89] and Horie et al.[90] have developed methods for V. cholera, but these methods are not very efficient for measuring the low densities of Vibrio that are found in marine environments. Horie et al.[91] developed a membrane filter technique for enumerating V. parahaemolyticus in 1967. The medium employed arabinose, ammonium sulfate, sodium cholate and a high pH (8.6). Baross and Liston,[92] Twedt and Novelli[93] and Vanderyant and Nickelson[94] developed media for V. parahaemolyticus but the utility of the media was limited because large volumes of water could not be tested. In 1976, Watkins et al.[95] developed a membrane filter enumeration technique for V. parahaemolyticus. Selection of V. parahaemolyticus was based on the ability of this organism to

grow in 3% NaCl, at high pH (8.6) and at 40°C. In situ galactose and sucrose fermentation tests, along with the oxidase test, were used to differentiate other species and verify V. parahaemolyticus colonies.

Vibrio species are similar to other aquatic bacteria in that they are ubiquitous in marine environments, and therefore, no special significance can be placed on detecting them in marine waters. The advantage of being able to isolate Vibrio species from water samples is that the source of this organism can be identified when it is suspected of causing disease or infections.

I. Legionella Species

Legionella pneumophila is the most important of the Legionella species because of its frequent association with respiratory illness. Although many other species have been identified since 1976 when L. pneumophila was first discovered,[96] these other species have not gained the importance of L. pneumophila. Since L. pneumophila has this position of importance, most of the media developed to isolate Legionella species have used it as the target bacterium. Presently three media are available for isolating Legionella species. All three use the charcoal, yeast extract, cysteine, ferric pyrophosphate agar base developed by Feeley et al.[97] Bopp et al.,[98] Wadowsky and Yee,[99] and Edelstein[100] modified the buffered basal medium by adding various antibiotic inhibitors. Bopp et al.[98] added cephalothin, colistin, vancomycin and cyclohexamide to control or eliminate non-Legionella bacteria found in water samples. Wadowsky and Yee[99] used glycine, vancomycin and polymyxin B to inhibit background flora. Edelstein[100] added anisomycin, bromthymol blue and bromocresol purple

to Wadowsky and Yee's modification of the basal medium. The efficiency of the media for eliminating background flora and recovering Legionella have been examined by Calderon and Dufour.[101] The modification developed by Edelstein was the most efficient of the three media for recovering Legionella species seeded into environmental water samples. Edelstein's modification was also the most efficient of the three media for isolating Legionella species from natural samples obtained from plumbing systems and from lakes and rivers. The background flora of water samples can be further reduced by acidifying the water sample to pH 2 for 10 minutes and then neutralizing the sample to pH 7.[97]

The three isolation media are usually used as plating media and when used in this manner, they have the disadvantage of not being able to assay large volumes of sample. The Communicable Disease Centers[102] recommends a method which involves membrane filtration of water samples. This technique allows much larger volumes of sample to be examined. After a water sample has been passed through the filter the membrane is placed grid side down in a flask containing 10 ml of the sterile filtrate and the flask is then placed into a sonic bath to release the concentrated bacteria from the membrane. The bacterial suspension then can be inoculated onto any of the above media, or it can be acid treated and then inoculated onto the medium.

The significance of isolating Legionella species, especially L. pneumophila, from water samples is not clear-cut. Legionella species appear to be ubiquitous in the aquatic environment, and therefore, their detection is of little consequence except when the water is to

be used for cooling or other purposes, such as showering. If the water is to be used for either of these purposes, there should be some concern because this organism can be amplified in hot water tanks or heat exchange systems and then dispersed as an aerosol. Some investigators believe that detecting Legionella in water samples, in the absence of overt disease in the exposed population, is not justification for taking action to eliminate the organism. Others believe that immediate action should be taken to eliminate Legionella from the water delivery system. However, in situations where high risk populations, such as intensive care wards in hospitals, and hospital patients taking immunosuppressive drugs, are exposed there is no disagreement. There is general agreement that steps chould be taken to eliminate Legionella if it is found in water to which these groups are exposed.

J. Chromobacterium

Chromobacterium violaceum is found in soil and water, particularly in tropical and subtropical regions. This bacterium which causes severe illness and even death is the most important health-related species of the genus Chromobacterium.[103] The number of cases associated with this organism, fortunately, is very limited.[103]

Several media, Bennett's agar,[104] BAN,[105] Ryall and Moss selective media[106] and Aeromonas membrane agar without indicator,[107] have been reported for the isolation of Chromobacterium from water. Bennett's medium is nonselective and was originally developed for the production of aerial mycelia from streptomyces. BAN medium is Bennett's agar with selectivity provided by the addition of neomycin hydrochloride, cyclohexamide and nystatin. The selective medium of

Ryall and Moss contains one-fourth strength nutrient agar and the selective agents colistin, sodium deoxycholate and cyclohexamide. The membrane Aeromonas medium is that described by Rippey and Cabelli[78] without the pH indicator, but containing ampicillin and sodium desoxycholate as inhibitors. Identification of members of the genus Chromobacterium can be made using the cultural and biochemical tests of Leifson,[108] and Sneath.[109,110]

Kobarger and May[107] compared Bennett's agar, Ryall and Moss medium and membrane filter Aeromonas agar incubated at 25°C and 35°C for the recovery of Chromobacterium species from foods, soil and water. The Aeromonas medium incubated at 35°C gave the highest recovery of C. violaceum. They reported C. violaceum only from soil and water. The nonpathogenic C. lividum was recovered from foods, soil and water.

The presence of C. violaceum in water appears to be limited to tropical and subtropical regions. This genus favors warm waters and its presence may be seasonal in nature. Based on the small number of reported cases, it appears to be of limited health hazard potential.

K. Leptospira

The isolation of Leptospira from surface waters is extremely difficult because these organisms grow very slowly, and therefore, they can be overgrown by competing bacteria. This difficulty is overcome by prefiltering the sample. Leptospira are commonly found in sediments of ponds and streams, and therefore, the first step in isolating this organism is to prefilter the sample through Whatman No. 1 filter paper to remove gross particulates. This step is followed by filtration through membranes of smaller porosity, the

minimum pore size usually being 0.45 microns.

The filtrate may then be cultured using several methods. Braun et al.[111] proposed that 0.2 µl and 2.0 ml volumes be inoculated into tubes of Fletcher's semi-solid medium containing 10% rabbit serum. The medium is incubated at 30°C for six weeks. Tubes are examined weekly using dark field illumination to determine if Leptospira are present. Medium in tubes that are positive for Leptospira is passed through a 0.22 micron membrane filter and the filtrate inoculated once again into tubes of Fletcher's medium, which are incubated at 30°C. Cultures that are not contaminated are used to determine the serotype and pathogenicity of the isolated strain.

Baseman et al.[112] recommend placing a drop of the filtrate in the center of each plate of serum agar or SM agar and these plates are incubated at 30°C. Growth appears in 7-9 days as thin veils moving to the periphery of the plate. Leptospira were transferred from the veils to other plates and streaked for isolation. Purified cultures may be serotyped and the pathogenicity of the strains determined.

Pathogenic and saprophytic isolates can be differentiatd in a number of ways. Johnson and Rogers[113] showed that 8-azaguanine inhibits the growth of pathogenic Leptospira and allows the growth of saprophytic strains. Johnson and Harris[114] examined the growth characterisitics of pathogenic and saprophytic Leptospira at low incubation temperatures. They found that the minimal growth temperature for pathogenic Leptospira was between 13 and 15°C, whereas saprophytic Leptospira have minimal growth temperatures between 5 and 10°C. Thus, saprophytes can easily be differentiated from pathogens by

incubating the cultures at 13°C in a medium containing 10% rabbit serum. The saprophytic strains grow at this temperature and the pathogenic strains cannot. Füsi and Csóka[115] found that the growth of pathogenic Leptospira is inhibited by the presence of 10 mg/L of copper sulfate in the culture medium, whereas saprophytic strains grew well.

The finding of pathogenic Leptospira in water samples from "swimming holes" or other surface waters used for swimming is a clear indication that the water is not suitable for bathing. Furthermore, it also indicates that domestic or wild animals sharing the water may be infected with Leptospira.

L. Enterotoxigenic Escherichia coli

Enterotoxigenic E. coli[116,117] produce illness through the production of heat labile (LT) and heat stable (ST) enterotoxins, cellular invasiveness, and heavy colonizatin of the intestinal mucosa.

These E. coli are specifically related to fecal contamination of water by warm-blooded animals and when found in water, represent a health risk for individuals using the water.

Enterotoxigenic E. coli cannot be differentiated from other E. coli unless they are tested for their effect on Y-1 adrenal and Chinese hamster ovary tissues, or with the suckling mouse, rabbit ileal loop or Sereny tests.[116] The development of specific genetic hybridization probes[118] may simplify the test for Enterotoxigenic E. coli.

The methods available for detection of enterotoxigenic E. coli include the MPN and membrane filter methods described in Standard Methods for the Examination of Water and Wastewater.[2] However, the

isolates from these methods must first be identified as E. coli by biochemical testing and then tested by previously described methods to determine their enterotoxigenicity. These methods are time-consuming and expensive. Furthermore, since the enterotoxigenic mechanism may be plasmid-controlled, these characteristics can be lost during the identification procedure.

Calderon and Levin[119] modified the E. coli membrane filtration method described by Dufour et al.[13] to isolate and enumerate enterotoxigenic E. coli (ETEC) from water. ETEC colonies on the modified medium were identified by transferring the membrane to Y-1 adrenal cell monolayers. E. coli producing LT toxin in approximately two days caused the Y-1 adrenal cells to round up. This method was successfully tested using fecal samples and polluted marine water samples. Although this method is the most facile one available, its usefulness is limited because it requires costly tissue culture techniques.

The detection of enteropathogenic E. coli in potable water indicates that there is a considerable risk for persons who drink the water and that some action to eliminate these organisms should be taken.

M. Giardia, Entamoeba histolytica and Balantidium coli

Giardia are protozoan flagellates which cause diarrhea in humans and have been commonly identified in waterborne disease episodes.[120] The presence of this parasite in humans usually is detected by the examination of fecal specimens by the microscopic examination of flotation or sedimentation concentrates. Microscopy also can be employed to identify Giardia cysts in water samples after concentra-

tion from the water. This organism was recovered initially from potable water during an outbreak in Rome, New York.[121] Methodology for the successful concentration, isolation and identification of Giardia from tap water using a depth filter are described by Jakubowski and Ericksen.[122] Holman et al.[123] compared 1 µm and 7 µm porosity yarn-wound depth filters for recovery of as few as 6,000 Giardia cysts per test, and determined that the 1 µm porosity depth filter exhibited higher cyst recovery rates. These investigators also compared membrane filtration and centrifugation methods for cyst recovery from yarn-wound filter concentrates and reported that higher numbers of cysts were recovered by centrifugation.

A workshop on Giardia methodology was held at the U. S. Environmental Protection Agency Laboratory in Cincinnati, Ohio in September of 1980. The recommended methdology for Giardia detection from potable water derived from this meeting has been described by Jakubowski.[124] The low efficiency of detection methods, relatively high cost of procedures and lengthy time for examination limit the use of present Giardia methodology for routine monitoring. However, these methods can be employed in suspected waterborne disease outbreaks where Giardia is the suspected etiologic agent.

Entamoeba histolytica causes diarrhea. Chang and Kabler[125] suggested the use of membrane filter procedures for the concentration and identification of Entamoeba histolytica from non-turbid waters. This organism is identified by microscopic examination of the water concentrate. The identification of E. histolytica trophozoites and/or cysts requires expertise in parasitological identification.

Chang and Kabler[125] also recommended the cultivation of samples

or sample concentrates into liver infusion medium. This methodology is presented in Standard Methods for the Examination of Water and Wastewater, 15th Edition.[2] Examination of water for E. histolytica probably should be limited to those waters involved in waterborne disease outbreaks where this protozoan is the suspected etiologic agent and should not be employed for routine monitoring.

Balantidium coli is the only ciliate pathogenic for man and exhibits worldwide distribution.[126] A waterborne outbreak of 100 cases was reported in Micronesia in 1971.[127] This organism rarely is found in specimens from patients in the United States. B. coli is isolated and identified from fecal specimens using clinical procedures. The protozoan can be cultivated in Barret and Yarbrough's Medium.[126] Since this ciliate is the largest protozoan to infect humans, the use of membrane filtration procedures as outlined for Entamoeba histolytica of Chang and Kabler[125] might be appropriate for the concentration, isolation and identification of this organism from non-turbid waters. Waters should be examined for this organism only if it is the etiologic agent in a waterborne outbreak and routine monitoring would be superfluous.

IV. SUMMARY

The literature describing the enumeration and isolation of bacteria is replete with methods used to measure water quality and detect pathogens, and new methods are continuously being developed. The limited number of methods described in this chapter were selected because they have been successfully used to monitor water quality or to detect pathogens associated with water contact diseases. Some are included because they have characteristics that fulfill a unique purpose, i.e., high volume enumeration techniques for

measuring bacteria that normally occur in water at very low densities. None of the methods are ideal in the sense that they are free of limitations, but any shortcomings they might have are not great enough so as to preclude their use as tools for measuring bacteria in water environments.

The bacterial indicator methods, if used properly, are an effective means of monitoring water quality and thereby protecting groups at risk against pathogens associated with fecal material from humans and other warm-blooded animals. The use of fecal indicator bacteria are most efficient when they are used to index the potential presence of pathogens, especially viral pathogens, that are unculturable or very difficult to culture. Norwalk virus, rotavirus and hepatitis-A virus fall into this category. However, one should be mindful that indicator bacteria are not effective in all situations. For instance, Giardia cysts, which contaminate water as a result of the deposition of human or animal fecal wastes have been found in potable water in the absence of indicator bacteria that warn of the presence of fecal contamination.

The methods that isolate pathogens and opportunistic pathogens from water samples are most useful for identifying the source of these organisms during disease outbreaks associated with water contact. From time to time proposals have been introduced to monitor environmental waters for the pathogens themselves. Two factors discourage the acceptance of proposals to routinely monitor for pathogens. First, pathogens occur sporadically in water and the presence of one pathogen is usually unrelated to the presence of other pathogens and, second, the isolation of bacterial pathogens is usually difficult because of their fastidious nutritional requirements. In the case of viruses, the logistically complex concentration and tissue culture methods discourage their use for routine monitoring. These factors preclude

the use of pathogen isolation methods for any role in water microbiology other than tracing pathogens to their source, so that corrective measures may be taken to eliminate the health hazard.

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